Effect of Melatonin on the Regulation of Proenkephalin and Prodynorphin mRNA Levels Induced by Kainic Acid in the Rat Hippocampus

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ABSTRACT: The in vivo short-term effect of melatonin on kainic acid (KA)-induced proenkephalin (proENK) or prodynorphin (proDYN) mRNA, and on AP-1 protein levels in the rat hippocampus, were studied. Melatonin (5 mg/kg) or saline was administered intraperitoneally (i.p.) to rats 30 min prior to and immediately after i.p. injection of KA (10 mg/kg). Rats were sacrificed 1 and 3 h after KA injection. The proENK and proDYN mRNA levels were significantly increased 3 h after KA administration. The elevations of both proENK and proDYN mRNA levels induced by KA were significantly inhibited by the preadministration with melatonin. The increases of proENK and proDYN mRNA levels induced by KA were well-correlated with the increases of c-Fos, Fra-2, FosB, c-Jun, and JunB protein levels, which were significantly increased 3 h after KA administration and effectively inhibited by administration with melatonin. In an electrophoretic mobility shift assay, both AP-1 and ENKCRE-2 DNA binding activities were increased by KA, which were also attenuated by the administration of melatonin. In addition, cross-competition studies revealed that AP-1 or ENKCRE-2 DNA binding activity was effectively reduced by the 50X unlabeled cross-competitor. Therefore, these data suggest that melatonin has an inhibitory role in KA-induced gene expression, such as proENK and proDYN mRNA expression, and this may be due to a reduction of KA-induced AP-1 or ENKCRE-2 DNA binding activity. *Hippocampus 2000;10:236–243. © 2000 Wiley-Liss, Inc.

KEY WORDS: AP-1; opioid; mRNA level; c-Fos; c-Jun

INTRODUCTION

In the mammalian pineal gland, melatonin is synthesized from serotonin and secreted into the blood under the influence of diurnal rhythm: higher levels of melatonin are found during periods of dark than during periods of light (Reiter, 1991a). In vivo, melatonin crosses the blood-brain barrier (Reiter, 1991b), is rapidly taken up by the brain (Menendez-Pelaez et al., 1993), and may exert a neurobiologically relevant action through interaction with specific G-protein-coupled receptors (Dubocovich, 1995; Reppert et al., 1995), with nuclear receptors (Menendez-Pelaez et al., 1993; Becker-Andre et al., 1994), or by scavenging reactive oxygen species (Tan et al., 1993; Reiter et al., 1994; Cagnoli et al., 1995) and preventing lipid peroxidation (Melchiorri et al., 1995).

The neuroprotective action of melatonin was first demonstrated in an in vitro model of glutamate receptor-mediated excitotoxicity in primary neuronal cultures (Giusti et al., 1996). This neuroprotective effect was selective for neurotoxicity triggered by kainic acid (KA)-sensitive glutamate receptors and did not involve direct glutamate receptor inhibition by melatonin. In vivo, KA administration to rats has been shown to result in epilepsy-like seizures that are accompanied by progressive neurodegeneration in different brain areas including the hippocampus, and KA-triggered excitotoxic brain damage is also reduced by intraperitoneal administration with melatonin (Giusti et al., 1996; Uz et al., 1996).

In addition to neurotoxic effects, KA can provoke the alternation of expression of several genes in the hippocampus, such as glial fibrillary acidic protein (Van Den Berg and Gramsbergen, 1993; Gramsbergen and Van den Berg, 1994), p53 (Sakhi et al., 1994), cytokines (Minami et al., 1991), and neurotrophic factors (Zafra et al., 1992; Bugra et al., 1994), which all have been suggested to be implicated in reactive gliosis (Van Den Berg and Gramsbergen, 1993; Gramsbergen and Van den Berg, 1994) and neuronal cell survival (Bugra et al., 1994; Sakhi et al., 1994; Pozas et al., 1997).

In addition, the excitation of the hippocampal neuronal pathways by KA administration increases proenkephalin (proENK) and prodynorphin (proDYN) and their immunoreactivity (Hong et al., 1980; McGinty et al., 1984; Kanamatsu et al., 1986). The amount of opioid peptide mRNA produced in the dentate gyrus is positively correlated with neuronal stimulation (Douglas et al., 1991), and may be regulated by the endocrinological environment, such as local concentrations of glucocorticoid and nitric oxide (Kim et al., 1997; Won et al., 1998). Although the exact functions of KA-induced opioid gene expression are not exactly known, the opioid
peptides are considered to be implicated in the modulation of hippocampal neuronal excitability (Hong et al., 1988; Lee et al., 1989).

Recently, we reported that KA-induced proENK and proDYN gene expression may depend on concomitant protein de novo synthesis, such as AP-1 (Fos/Jun) (Won et al., 1997). Indeed, both proENK and proDYN promoter/enhancer regions contain an identical AP-1-like domain, ENKCRE-2 (enkephalin CRE-2) or DYNCRE-3 (dynorphin CRE-3), which may play a critical role in the regulation of their gene expression through interaction with AP-1 and CREB proteins (Comb et al., 1986; Douglass et al., 1989; Sonnenberg et al., 1989a,b,c; Messersmith et al., 1994). However, in the rat hippocampus, the modulatory role of melatonin in the regulation of KA-induced opioid gene expressions has been unveiled. Therefore, in the present study, the effect of melatonin on the regulation of KA-induced proENK and proDYN mRNA expression in the rat hippocampus was examined. In an attempt to delineate the basic mechanism of melatonin in KA-induced opioid mRNA levels, proENK and proDYN mRNA levels and the expression of AP-1 proteins and their DNA binding activity to AP-1 or ENKCRE-2 DNA motif were examined.

Materials and Methods

Animals and Drug Administration

Male Sprague-Dawley rats weighing 220–260 g were used. Animals were housed 2 per group in a room maintained at 22 ± 3°C with an alternating 12-h light-dark cycle. Animals were used only once. Melatonin (2.5 mg/ml), which was prepared in 5% ethanolic saline or an equal amount of vehicle, was administered intraperitoneally (volume 0.2 ml/100 g), 30 min before and immediately after KA administration (total cumulative melatonin dose, 10 mg/kg). KA was dissolved in phosphate-buffered saline (10 mM phosphate buffer, 2.7 mM KCl, 137 mM NaCl), adjusted to pH 7.5 with 1 N NaOH, and administered intraperitoneally at a dose of 10 mg/kg. Animals were sacrificed 1 and 3 h after KA administration.

Isolation of Total RNA and Proteins

Total cellular RNA and proteins were extracted from the rat hippocampus, using a rapid guanidine thiocyanate-water saturated phenol/chloroform extraction, and subsequent precipitation with acidic sodium acetate. Total cellular RNA in the aqueous phase was precipitated with cold isopropanol alcohol. Isolated RNA samples were subjected to spectrophotometric analysis at 260 nm and 280 nm. The separated organic layer was extracted twice with an equal volume of sterile Millipore water, and proteins were precipitated by adding two volumes of absolute ethanol to the water-extracted organic phase. The protein pellets were washed twice with cold absolute ethanol and dried. The dried pellets were dissolved in a denaturing buffer (6 M guanidium chloride, 20 mM Tris-HCl, pH 8.0, and 1 mM EDTA), and were dialyzed against a renaturing buffer (20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA, 5 mM dithiothreitol, 5 mM MgCl₂, 0.4 mM phenylmethylsulfonyl fluoride, and 20% glycerol) at 4°C. The concentration of protein was determined with the Coomassie blue protein assay reagent (Pierce Chemical Co., Rockford, IL), using bovine serum albumin as a standard.

Nonisotope Northern Blot Hybridization Analysis

Ten micrograms of total RNA were denatured and electrophoresed on 1% agarose-formaldehyde gels and transferred to nylon membranes (Amersham, Buckinghamshire, UK). After ultraviolet cross-linking, the membranes were prehybridized at 68°C for at least 1 h in prehybridization buffer (5 × SSC, 50% formamide, 0.02% SDS, 0.1% sodium N-lauroyl sarcosine, 2% blocking reagent; Boehringer Mannheim, Mannheim, Germany). The DIG-labeled proENK and proDYN probes were added to prehybridization buffer containing 50% formamide. The membranes were incubated overnight at 68°C in a shaking water bath, and were washed twice for 10 min per wash in 2 × wash solution (2 × SSC, 0.1% SDS) at room temperature. Then, the membranes were washed twice for 15 min per wash in 0.1 × wash solution (0.1 × SSC, 0.1% SS). After equilibrating in buffer I (100 mM maleic acid, 150 mM NaCl, pH 7.5) for 1 min, the membranes were gently agitated in buffer II (1% blocking reagent in buffer I) for 30–60 min. The membranes were hybridized with the diluted anti-DIG-alkaline phosphatase (1:10,000 (75 mU/ml), Boehringer Mannheim) in buffer II for 30 min. After washing the membranes twice for 15 min per wash in 0.3% Tween-20 (in buffer I), the membranes were equilibrated in buffer III (100 mM Tris-HCl, pH 9.5, 100 mM NaCl, and 50 mM MgCl₂) for 2 min. Diluted CSPD™ (Boehringer Mannheim) (1:100 dilution in buffer III) was spread over the surface of the membranes. After incubation of the membranes at 37°C for 15–20 min, the membranes were exposed to HyperFim-ECL (Amersham) for detection of the chemiluminescent signal. For rehybridization, blots were washed for 20 min at room temperature in sterilized (Millipore) water, and then the membranes were washed overnight at 65°C in 50 mM Tris-HCl (pH 8.0), 50% dimethylformamide, and 1% SS to remove the hybridized probe. Blots were rehybridized to the DIG-labeled rat cyclophilin cRNA probe, a gene encoding peptidyl-prolyl cis-trans isomerase, which is constitutively expressed in most mammalian tissues with the exception of skeletal muscle (Danielson et al., 1988; Takahashi et al., 1989). The cRNA probes for proENK (Yoshikawa et al., 1984), proDYN (Cievelli et al., 1985), and cyclophilin (Danielson et al., 1988) were synthesized in vitro from linearized expression vectors with DIG-UTP, as suggested by the manufacturer (Boehringer Mannheim).

Western Immunoblot Analysis

Total cellular protein (50 μg) was separated by electrophoresis in 12% polyacrylamide gels. A prestained rainbow protein mixture (Amersham Co., Arlington Heights, IL) was used as the molecular weight standard. Electrotransferred polyvinylidene difluoride, filters were first blocked with blocking buffer (3% skim milk, 1%
BSA, 10 mM Trizma base, pH 8.0, and 150 mM NaCl) and then incubated with antisera against c-Fos (1:1,000; Santa Cruz Biotechnology, Santa Cruz, CA), Fra-2 (1:1,000; Santa Cruz Biotechnology), FosB (1:1,000; Santa Cruz Biotechnology), c-Jun (1:1,000; Santa Cruz Biotechnology), and JunB (1:1,000; Santa Cruz Biotechnology) in blocking buffer for 4 h at room temperature. Filters were then washed three times with Tris-buffered saline containing 0.3% Tween-20 (TBST; 10 mM Trizma base, pH 8.0, 150 mM NaCl, and 0.3% Tween-20) for 5 min and then incubated with the goat anti-rabbit donkey IgG-horseradish peroxidase conjugate (1:5,000) in blocking buffer at room temperature for 1 h. After washing the filters with TBST for 10 min (three times), ECL-plus solution (Amersham Life Science Co., Buckinghamshire, UK) was added. Then the membranes were exposed to Hyperfilm-MP (Amersham) for detection of light emission.

Nonisotope Electrophoretic Mobility Shift Assay

AP-1 (5'-CGC-TTG-ATG-CTG-CCG-GAA-3'; Santa Cruz Biotechnology) and ENKCRE-2 (5'-CTA-GTG-ATG-CGT-CAG-CCG-GAT-C-3'; Korea Biotech, Dae-Ku, Korea) were annealed by incubating an equal molar concentration of each single-stranded oligonucleotide in 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, and 200 mM NaCl at 95°C for 10 min, and then the mixture was allowed to cool to room temperature. The DNA-binding assay was performed by following the instructions in the manual provided by the DIG-Gel Shift Kit (Boehringer Mannheim). Binding reactions were carried out at room temperature for 20 min, and reaction mixtures contained 50 μg of total protein, 20 mM HEPES (pH 7.6), 30 mM KCl, 5 mM MgCl₂, 1 mM EDTA, 1 mM dithiothreitol, 0.2% Tween-20, 50 μg/ml poly (dI-dC), and approximately 0.3 pmol of specified probe labeled with DIG-ddUTP, using terminal deoxynucleotidyl transferase (Boehringer Mannheim). Protein-DNA complexes were separated from protein-free DNA by nondenaturing electrophoresis in 4% polyacrylamide (30:1, acrylamide:bisacrylamide) gels. Gels were run at room temperature in 89 mM Tris (pH 8.3), 89 mM boric acid, and 2 mM EDTA at constant voltage (8 V/cm), and were electrophoretically loaded onto positively charged nylon membranes. The membranes were baked at 80°C for 15 min, washed with 0.3% Tween-20 in buffer I, and hybridized with the diluted anti-DIG-alkaline phosphatase (1:10,000; 75 mU/ml) in buffer II for 30 min. After two washes for 15 min with 0.3% Tween-20 (in buffer I), the membranes were equilibrated in buffer III (100 mM Tris-HCl, pH 9.5, 100 mM NaCl, and 50 mM MgCl₂) for 2 min. The method for detection of chemiluminescence was identical to the method used for the nonisotopic Northern blot analysis.

Statistical Analysis

The levels of proENK and proDYN mRNA or c-Fos, Fra-2, FosB, c-Jun, and JunB proteins were quantified with the Bio-profil Bio-1D application (Vilber-Lourmat, France), and expressed as percentage of control proENK or proDYN mRNA levels. ANOVA and Newman-Keuls tests were used to identify statistical significance for multiple comparisons. Differences were considered significant at \( P < 0.05 \).

RESULTS

Effects of Melatonin on KA-Induced proENK and proDYN mRNA Levels in the Rat Hippocampus

All data shown in the present study were determined at least three times and showed the same tendency regarding results. The effects of melatonin on proENK and proDYN mRNA levels in rat hippocampus were examined at 1 and 3 h after KA administration.
As shown in Figure 1A, the proDYN mRNA level was slightly, but not significantly, increased 1 h after KA administration, and significant increases in proENK and proDYN mRNA levels were observed 3 h after KA administration (Fig. 1A,B). The proENK and proDYN mRNA levels were elevated about 6.4- and 4.2-fold, respectively, 3 h after KA administration. The elevations of both proENK and proDYN mRNA levels by KA were significantly inhibited by the administration of melatonin about 3.4- and 1.9-fold, respectively.

Effects of Melatonin on KA-Induced Fos-Family Protein Levels in the Rat Hippocampus

Western blot analyses using antibodies against c-Fos, Fra-1, Fra-2, and FosB proteins were carried out to examine whether there were correlations between opioid peptide mRNA expression and Fos-family protein levels (Fig. 2). The c-Fos, Fra-2, and FosB protein levels were not affected by KA, melatonin, or melatonin plus KA at 1 h after drug administration, but at 3 h after KA administration, the levels of c-Fos (about 10.2-fold) and Fra-2 (about 4.8-fold) began to increase. In addition, KA also increased the protein levels of both the 45-kDa-long form of FosB (FosB-L; about 21.5-fold), a completed encoded FosB, and the 35-kDa short form of FosB (FosB-S; about 17.6-fold), a truncated form of FosB (Nakabeppu and Nathans, 1991). Although administration of melatonin did not affect the basal levels of c-Fos, Fra-2, and FosB, it effectively reduced KA-induced increases of c-Fos (about 7.4-fold), about 4.8-fold, and JunB (about 2.1-fold) protein levels.

Effects of Melatonin on KA-Induced Jun-Family Protein Levels in the Rat Hippocampus

Western blot analyses using antibodies against c-Jun and JunB proteins were also carried out (Fig. 3). The protein levels of c-Jun and JunB were not affected by KA, melatonin, or melatonin plus KA at 1 h after drug administration, but at 3 h after KA administration, the levels of c-Jun (about 4.4-fold) and JunB (about 4.3-fold) began to increase, but not JunD. The administration of melatonin also effectively reduced KA-induced increases of c-Jun (about 7.4-fold) and JunB (about 2.1-fold) protein levels.
Effects of Melatonin on KA-Induced AP-1 or ENKCRE-2 DNA Binding Activity in the Rat Hippocampus

As revealed by the electrophoretic mobility shift assay, both AP-1 and ENKCRE-2 DNA binding activities began to increase 3 h after KA administration (Fig. 4). The increases of AP-1 and ENKCRE-2 DNA binding activities induced by KA were inhibited by the preadministration of melatonin. The reductions of AP-1 and ENKCRE-2 DNA binding activities by self-competition with 50 × excess of unlabeled (cold) probes showed that these binding activities were specific (Fig. 4C). The cross-competitions between AP-1 and ENKCRE-2 showed that AP-1 or ENKCRE-2 DNA binding activity was diminished by 50 × unlabeled cross-competitor. These results imply that the ENKCRE-2 element behaves like an AP-1 element.

**FIGURE 3.** Effect of melatonin on c-Jun (A) and JunB (B) protein expression induced by kainic acid (KA) in rat hippocampus. Melatonin (mel, 5 mg/kg) or equal amounts of vehicle (CON, 5% ethanolic saline) were administered intraperitoneally 30 min before and immediately after KA administration. After i.p. administration of KA or PBS, animals were sacrificed at indicated time points (1 and 3 h). Fifty micrograms of total cellular proteins, which were extracted from pooled rat hippocampus (n = 3/group), were used for determination of c-Jun and JunB protein levels using Western immunoblot analysis. A polyclonal antiserum against c-Jun or JunB proteins was used at a 1:1,000 dilution. In c-Jun and JunB panels, 39-kDa bands indicate c-Jun and JunB bands respectively. Vertical bars in the column graph indicate SE. *P < 0.05, **P < 0.01 compared to the control group; +P < 0.05 compared to the KA-administered group. n = 3 independent experiments.

Effects of Melatonin on KA-Induced AP-1 or ENKCRE-2 DNA Binding Activity in the Rat Hippocampus

In the present study, we demonstrated that administration of melatonin to rats may reduce KA-induced proENK and proDYN mRNA levels via reducing the expression of AP-1 proteins and their DNA binding activity to the ENKCRE-2 (or DYNCRE-3) DNA domain. The exact role of the inhibitory mechanism of melatonin on KA-induced opioid mRNA levels is not understood. One possible mechanism is the action of melatonin as an anticonvulsant. Although this study did not provide behavioral data in relation to the effect of melatonin on KA-induced convulsions, a recent study suggested that, in mice, intracerebroventricular (i.c.v.) pretreatment with melatonin attenuates the convulsant effect of i.c.v.-administered KA, as well as quinolinic, glutamate, N-methyl-D-aspartate, and pentylentetrazole (Lapin et al., 1998). Furthermore, in rats, although melatonin did not completely abolish KA-induced convulsions, it significantly reduced KA-induced convolution severity (Giusti et al., 1996). Because KA-induced increases of proENK and proDYN mRNA levels are positively correlated with seizure intensity (Douglass et al., 1991), reduced seizure activity due to melatonin may be directly responsible for the inhibition of KA-induced opioid peptide mRNA levels. Although the exact mechanisms underlying the anticonvulsant effect of melatonin in the KA-induced limbic seizure model are not understood, melatonin is believed to influence inhibitory GABAergic neurotransmission (Golombek et al., 1996). Indeed, such an action of melatonin might interfere with KA-induced excitotoxicity and reduce toxic oxidative damage through a mechanism that does not require reactive oxygen species scavenging (Kennaway et al., 1988). Since drugs which are known to enhance GABA-mediated neurotransmission, such as diazepam, pentobarbital, and valproic acid, block both seizure activity and increases in the AP-1 binding factor (Sonnenberg et al., 1989a,b; Pennypacker et al., 1993), the anticonvulsant effect of melatonin may reduce the activation of KA-induced c-Fos, Fra-2, c-Jun, and JunB expression as well as their DNA binding activity to the AP-1 DNA motif. The promoter/enhancer region in proENK or proDYN contains an identical AP-1- or CRE-like sequence called ENKCRE-2 (enkephalin-CRE) or DYNCRE-3 (dynorphin-CRE) (Comb et al., 1986, 1988; Messersmith et al., 1994), which is believed to play an important role in the regulation of these expressions. Affinity-purified AP-1 transcriptional factors bind to the ENKCRE-2 sequence (Comb et al., 1988), and in vitro transfection of c-jun and c-fos transcripts activates transcription of a reporter gene containing the ENKCRE-2 element (Sonnenberg et al., 1989c), suggesting that AP-1 transcriptional factors actively participate in proENK gene expression. However, in striatal nuclear extracts, 30-fold excess AP-1 did not cross-compete with ENKCRE-2...
Konradi et al., 1993), suggesting that ENKCRE-2 activity can be modulated by other transcriptional factors such as CREB. In the present study, AP-1 or ENKCRE-2 DNA binding activity was effectively attenuated by 50 \times \text{unlabeled cross-competitor}, suggesting that ENKCRE-2 in KA-treated hippocampus may be modulated by the AP-1 transcriptional factor rather than CREB. Indeed, our recent study reported that KA-induced increases of both AP-1 and ENKCRE-2 DNA binding activities were effectively reduced by addition of antiserum against Fos- or Jun-family proteins, and that the increases of proENK and proDYN mRNA levels depended on the concomitant protein de novo synthesis, such as AP-1 (Fos/Jun) (Won et al., 1997). Therefore, these results suggest that the inhibition of KA-induced AP-1 expression by preadministration of melatonin may result in a reduction of KA-induced increase of ENKCRE-2 DNA binding activity and a subsequent reduction of proENK and proDYN mRNA levels.

In addition to the role of melatonin as an anticonvulsant, several lines of evidence in in vivo and in vitro experiments have shown that the melatonin acts as a scavenger against reactive oxygen species (Tan et al., 1993). Indeed, the formations of reactive radical species were reported to be involved in AP-1 activation (Pinkus et al., 1996; Rong and Baudry, 1996). Furthermore, in the experiment with the Cu/Zn superoxide dismutase-overexpressing transgenic mouse, superoxide radicals were reported to modulate c-fos and c-jun expression after KA-induced seizure (Kondo et al., 1997). Therefore, although the activation of AP-1 and opioid gene expression in the seizure rat has been predominantly believed to result in an excitability of hippocampal neurons, there is a possibility that melatonin may reduce AP-1 and opioid gene expression via scavenging the reactive radicals. The studies related to this possibility should be performed in the future.

Although several previous studies suggested AP-1 proteins are a major factor in the regulation of proENK gene expression, there were several lines of evidence that certain factors such as AP-2, AP-4, NFκB, and NF-1 also bind to the proENK promoter region and, in turn, regulate the expression of the proENK gene (Comb et al., 1988; Hyman et al., 1989; Rattner et al., 1991). In addition, in proDYN gene expression, NFκB has also been suggested to have a crucial role (Bakalkin et al., 1994), as well as AP-1/CREB (Messersmith et al., 1994). Indeed, NFκB is regarded as a transcriptional factor which reflects the cellular redox potential (Pinkus et al., 1996). Furthermore, seizure activity results in a rapid induction of NFκB in the adult rat hippocampus (Rong and Baudry, 1996). Therefore, the molecular mechanisms involved in proENK and proDYN gene expression may not be as simple as recognition of enhancer domains by AP-1. In addition, more complicated second messenger systems that are influenced by melatonin may also be involved in the regulation of opioid transcription. The exact nature of the interactions between these transcription factors and the opioid peptide gene in the hippocampus needs to be further determined.

In conclusion, we have demonstrated that melatonin inhibits the expression of proENK and proDYN mRNA levels in the KA-induced limbic seizure model. In addition, melatonin also suppressed KA-induced expression of AP-1 proteins, such as c-Fos, Fra-2, FosB, c-Jun, and JunB, which are believed to be involved in the regulation of proENK or proDYN mRNA expression.
REFERENCES


